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EFFECT OF PHYSICAL ACTIVITY ON ATHEROGENESIS

AN EXPERIMENTAL STUDY IN COCKERELS

BY

ESKO J. ORMA

Vammala 1957

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PREFACE

The present study was carried out at a time when the Institute of Physiology was undergoing complete renovation. Under these circumstances I was obliged to approach the chiefs of many other institutes for working facilities. All the persons I had to trouble, showed me the greatest kindness.

The study was carried out under the direction of *M. J. Karvonen,* M.D., Ph.D., Docent in Physiology, University of Helsinki, and Director of the Physiological Department at the Institute of Occupational Health, Helsinki. My discussions with him were of decisive importance in encouraging me to undertake this work. He followed and guided my work with unfailing interest and I have the greatest pleasure in here expressing my thanks to him.

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by him at his Clinic and for the encouragement and advice I received from him during the final phase of the work.

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I wish also to thank the personnel of the Institute of Physiology, of the Statistical Section of the Institute of Occupational Health, of the Clinical Laboratory at the Second Medical Clinic and of the Medical Outpatient Department, University of Helsinki for their assistance and patience.

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Helsinki, April 1957

E. O.

Introduction

Some investigations of the last few years have suggested that coronary heart disease is more rare and milder in type in the physically active than in inactive persons. Experimental studies have shown that physical activity is also able to prevent elevation of the plasma cholesterol level, often associated with atherosclerosis. These findings attract serious attention to a possible correlation between atherogenesis and physical activity.

Physical Activity and Coronary Heart Disease

The investigation conducted by the Social Medicine Research Unit of the Medical Research Council of Great Britain under the direction of *J. N. Morris* into the occurrence of coronary heart disease in certain groups of weekly paid employees of the London Transport Executive, in postal workers and Civil Servants (*Morris* et al. 1953 a) was the first study to pay attention to the possible correlation between atherosclerosis, or at least between the occurrence of coronary heart disease, and physical activity. The population of transport workers included about 31,000 men aged 35 to 64 who represented the following occupations: drivers and conductors of the double-deck buses, drivers and conductors of trains and trolley buses, motormen and guards on the underground railway. The investigation took into account only the first absence from work because of coronary heart disease during 1949 and 1950. It was regarded as the first clinical episode and the incidence in this study means

the rate (per 1,000) of occurrence of first clinical episodes. The investigation showed drivers to have a higher incidence and more serious form of coronary heart disease than conductors. The total incidence was 2.7 in drivers, 1.9 in conductors; immediate deaths (in the first three days) accounted for 31 per cent of the first episodes in drivers, 19 per cent in conductors.

These results interested investigators in the possibility that the physical effort involved in a conductor's work might be a protective factor, safeguarding this group from some of the most severe manifestations of coronary heart disease suffered by less active workers. To study this hypothesis, a population of about 100.000 postal men and Civil Servants were investigated by the same method. They were classified according to the activity in their occupations into three categories: active (postmen), intermediate, and sedentary. The findings resembled those made regarding transport workers; the postmen showed a lower incidence and less severe manifestation of coronary heart disease than the less active groups. The intermediate group lay, on the whole, between the postmen and the sedentary categories.

On the basis of these observations, *Morris* et al. (1953 b) introduced the following working hypothesis: »Men in physically active jobs have a lower incidence of coronary heart disease in middle age than have men in physically inactive jobs. More important, the disease is not so severe in physically active workers, tending to present first in them as angina pectoris and other relatively benign forms, and to have a smaller early case-fatality and a lower early mortality rate». This hypothesis was tested. It was supported by, for example, the following additional observations. The early mortality-rate of conductors and postmen in 1951—52 was again lower than that of drivers and telephonists. An analysis made of the Registrar-General's occupational mortality data for 1930—32 showed that at 45—64 years of age heavy workers experienced half the mortality from coronary heart disease of light workers.

Similar findings have been made in investigations conducted elsewhere. *Biörck* et al. (1954) published a survey of the cases

of coronary heart disease and cardiac infarction admitted to the Department of Medicine, Allmänna Sjukhuset, Malmö, during the years 1934—1953. The study showed, in a comparison of the city's total population classified according to occupation, that cardiac infarcts occurred more frequently in sedentary workers. Manual workers accounted for 89 per cent of the total population whereas they represented 68 per cent of the infarct patients. Sedentary workers were 11 per cent of the total population but 32 per cent of the infarct patients. The more detailed classification of occupation according to the physical effort in work showed, e.g., that persons doing heavy manual work accounted for 33 per cent of the population but represented merely 5 per cent of the infarct patients.

Luonqo (1956) made an investigation in the United States with reference to this point. His material consisted of a group of 100 patients with manifest coronary disease and a control group of 200 people with the same distribution of ages and occupations but without coronary disease. It emerged that in the coronary group 70 per cent showed no regular exercise patterns either at work or away from the job, but only 30 per cent of the control subjects had no regular exercise patterns. *Simonson* (1957) compared the electrocardiograms taken while resting and after exercise of 150 railroad clerks (sedentary work) and 150 switchmen (physical work). When the men with arterial hypertension were eliminated, the incidence of electrocardiographic abnormalities was significantly lower in the switchmen than in the clerks. According to the author, this speaks for a lower degree of coronary atherosclerosis in the switchmen.

The investigations cited would seem to indicate that ischemic heart disease is more common in people doing light work. On the other hand, *Master*'s extensive study (1941) based on a series of 1,700 infarct patients did not support this idea, since the occupational distribution of the patients did not deviate from the general occupational distribution of the population.

Experimental Studies of the Effect of Physical Activity on Atherogenesis

Wolffe et al. (1949) found that the incidence of atheromatosis and atherohepatosis was considerably lower in wild ducks than in domesticated ducks and geese. As one explanation for this they suggested the difference in physical activity. Employing forced-fed geese as experimental animals, the investigators observed the reversibility of atheromatosis and atherohepatosis e.g. by the use of exercise. From a later paper (*Wolffe* et al. 1952) it appeared that in two of eight geese which were individually confined in small cages slight atheromatosis appeared, but there was no atheromatosis in any of eight geese not confined. Both groups were fed with common poultry feed. The authors suppose that even confinement may cause the atheromatous syndrome.

Brown et al. (1956) studied the effect of exercise on cholesterol-induced atherosclerosis in rabbits. Two different atherogenic diets were used. The one with 0.1 per cent of cholesterol produced moderate and the other with 0.5 per cent considerable atherosclerosis in the experimental animals. Compulsory exercise was given each day in a large cylindrical barrel turning on a horizontal axis at 12—15 rounds per minute; two experimental series were exercised 20 minutes at a time per day, a third series for 60 minutes in all divided into three parts per day. In the experiment to study the effect of exercise on the development of atherosclerosis the animals were killed when the experiment had lasted 8 and 12 weeks. In the experimental series concerned with the disappearance of cholesterol from the vessels once the atheromata were developed the animals were kept on an atherogenic diet for 12 weeks and killed after a recovery period of 4, 8 or 10 weeks. The investigation established that exercise had no effect on either the development or the disappearance of atherosclerosis.

Wong et al. (1956) observed that exercise inhibited atherogenesis in the abdominal aorta of young cholesterol-fed chicks.

The diet contained 2 per cent of cholesterol. The birds were exercised twice daily, the whole experiment lasted eight weeks.

Physical Activity and Serum Lipids

Numerous observations support the assumption that atherosclerosis is associated with a disturbance of lipid metabolism. This, in spite of differences of opinion concerning details, is admitted e.g. by all the authors concerned in the Report of a Cooperative Study of Lipoproteins and Atherosclerosis (1956). And in experimental atherosclerosis the association between atherogenesis and pathologic plasma lipid values is especially distinct (*Katz* and *Stamler* 1953). It thus seems to be possible to obtain some information of the effect of physical activity on atherogenesis by studying the effect of physical activity on serum lipids.

Mann and his group in their epidemiological studies observed differences between the serum lipids of different population groups which they attributed to the differing physical activity of the groups. They found (*Mann* et al. 1955 a) that in rural Guatemalan subjects, by occupation chiefly manual workers with a diet containing only 8 per cent of calories from fat, the total cholesterol content of the plasma was considerably lower in all age classes than the plasma cholesterol of either urban Guatemalans or North Americans who were chiefly business and professional people with a diet including plenty of fat, 36—40 per cent of calories. But the beta lipoproteins (S_f 12—20 and S_f 35—100 classes) were only slightly lower in rural Guatemalan males than in North Americans, and in rural Guatemalan females frequently higher than in the North American group. Urban Guatemalans showed lipoprotein levels as high as or higher than those of the North Americans. A surprising feature of this study was the fact that although there was a considerable difference in the plasma cholesterol level between rural Guatemalans and North Americans the difference in lipoproteins was either rather small, as was the case with the men, or the con-

trary as was the case with the women. The investigators pointed out that such a combination, viz. low cholesterol content and high beta lipoprotein content is indeed rare. They established it in only 70 of the approx. 10,000 sera of North Americans studied by them. They were of the opinion that the fat content of the diet of the groups did not explain the differences in the lipid values since it did not permit an explanation of the dissociation of cholesterol and lipoprotein measurements. Investigations into the diet and into the weight and the weight-age changes of the group members showed the caloric expenditures of rural Guatemalans to be higher than those of the other groups. The investigators advanced the opinion that »the serum lipoprotein levels may be dependent upon the magnitude of energy turnover whereas the serum cholesterol levels are increased by energy accretion or fat deposition». A comparison of the levels of the S_f 12—20 and S_f 20—100 classes of lipoprotein and total serum cholesterol of 46 Nigerian men with those of an age- and weight-matched group of United States citizens led to a similar finding (*Mann* et al. 1955 b). The serum cholesterol content of the Nigerians was considerably lower than that of the Americans, but in beta lipoproteins the difference was small and of doubtful significance. The fat content of the diet of the Nigerians was low, 8—15 per cent of calories, but in this case too the authors did not regard the fat content of the diet as a significant factor and advanced the hypothesis that the low serum cholesterol of the Nigerians, like that of the Guatemalans investigated earlier, is caused by the muscular activity and energy expenditure required in those cultures. »This proposes that a large muscle mass or a large muscular expenditure is the effective agent, and that this, not the diet, is the important element in preventing hyperlipaemia and perhaps atherosclerosis».

They have tested this hypothesis by human experiments (*Mann* et al. 1955 c). The subjects were three male undergraduates who were put on a diet with a caloric content that was double that of their normal diet; the fat content, however, was kept the same. During this period the subjects' physical

activity was increased enough to consume the excess amount of dietary calories. After four weeks the increased physical activity was discontinued but the diet kept unchanged. Beta lipoproteins, phospholipids, total cholesterol and free cholesterol were observed in the investigation. It was found that as long as the increased activity really consumed the excess dietary calories the serum lipid values remained unchanged, but as soon as the subjects gained weight because of insufficient physical exercise the lipid values increased significantly in 2 of the 3 subjects. According to the authors, the *modus operandi* in the effect of physical activity on serum lipids, and also on atherogenesis, is that activity increases the expenditure of energy and prevents a positive caloric balance, a situation in which serum lipids, irrespectively of the nature of the diet, tend to rise, as e.g. *Walker* and his associates (*Walker* 1953, *Walker* et al. 1953) have shown.

Chailley-Bert et al. (1955) also, in their small material, observed that the total cholesterol level was higher in sedentary persons than in the more active, and *Keys* et al. (1956 b) found that activity inhibits the slight but nevertheless significant post-prandial increase in plasma cholesterol. *Keys* et al. (1956 a) also analysed in this sense their material consisting of populations with considerable differences, e.g. in dietary fat content. In each of these populations, representing different serum cholesterol levels, the subjects were distributed into three groups according to the estimated physical exertion they experienced. It was found that »within some populations there was a tendency for men in heavy manual labor to have somewhat lower serum cholesterol values than the other men in the population». On the other hand, the authors state that »differences in physical activity do not explain the large differences in serum cholesterol found when groups with different dietary habits are compared; the habitual diet, and especially its fat content, has much more influence than the physical activity, *per se*, on the concentration of total cholesterol and beta lipoprotein cholesterol in the blood serum».

Brown et al. (1956) in the investigation cited above observed the effect of a longer-term difference in activity on the serum cholesterol of rabbits. In the exercise groups the total serum cholesterol of rabbits maintained on a cholesterol diet was towards the end of the 12-week test only about a half of the total cholesterol content of the non-exercise groups. As the same result was achieved in the different test series the difference seems to be significant. *Peltonen* and *Karvonen* (1956), on the other hand, in their mouse experiments in which activity was increased by forcing the animals to swim, found no distinct change in the cholesterol level in the exercise groups whether kept on their habitual diet, a cholesterol diet or on a diet rich in fat. In the experiment carried out by *Wong* et al. (1956) in chicken exercise had no influence on the plasma cholesterol level of androgen-treated or cholesterol-fed birds.

The results of investigations concerning the effect of a short-term, vigorous physical exercise on serum cholesterol are conflicting. *Farig* and *Wacker* (1932) found that severe physical exercise raised the free and ester cholesterol levels by about a third, *Rakestraw* (1921) and *Patterson* (1927) found no change, and *Robinson* et al. (1927) established a fall in the levels. These experiments were made on men. As regards neutral fat and phospholipid, it has been found very generally that short-term severe exercise increases their content in blood (*Gage* ond *Fish* 1924, *Patterson* 1927, *Stewart* et al. 1931, *Fahrig* and *Wacker* 1932). These experiments also were made on men. On the other hand *Hiramatsu* (1932) found no change in serum lipids in rats maintained before exercise on a rice diet, but if the diet was especially rich in proteins the exercise caused a considerable drop in the serum lipids.

The Problems of the Present Investigation

The main sources of information thus far on the effect of physical activity on the development of atherosclerosis, epidemiological studies and investigations into the effect of physical activity on plasma lipids, provide circumstancial evidence only concerning the effect of physical activity on atherogenesis. Animal experiments concerning this problem have been made on a very small scale and the results are conflicting.

The epidemiological studies show only the difference, existing or non-existent, in the incidence and severity of coronary heart disease between the population groups of varying physical activity at work or outside work. In addition, they may indicate that physical activity affects one manifestation of atherosclerosis, the clinical manifestation of ischemic heart disease. They do not prove any correlation between physical activity and atherogenesis. In the first place, the groups compared may differ in many other respects, not only in the degree of physical activity. Secondly, the clinical manifestation of coronary heart disease depends largely on factors and disturbances which are independent in themselves of the severity of atherosclerosis, or only slightly dependent on it, e.g. on thrombosis formation, on the occurrence of intra-mural haemorrhages, on hemodynamic factors, on the general condition of the coronary arteries and the myocardium, etc. It may be worth mentioning that *Morris* (1955) does not consider at all that the results of the studies conducted by his group show any correlation between physical activity and atherosclerosis as such.

As regards the studies concerning the effect of physical activity on serum lipids, the circumstances are the same. It seems that physical activity decreases the level of serum cholesterol, at least in some circumstances, though this does not in any way prove that its effect on atherogenesis is similar.

In any case the observations to date certainly warrant continued studies of the effect of physical activity on atherogenesis *per se*. It seems, too, that the solving of the problem in its

present stage requires above all an experimental approach. Because there is no useful method of following atherogenesis and of measuring the degree of atherosclerosis in living human beings, the present author decided on animal experimentation.

The cockerel was chosen as the experimental animal since it has been used much in studying experimental atherosclerosis and there is consequently abundant information on atherosclerosis of this species. Since an increase in some plasma lipid values is generally considered to exist in atherosclerosis, and in experimental atherosclerosis in particular, total serum cholesterol, phospholipid, the distribution of cholesterol in the alpha and beta lipoprotein fractions, and the total cholesterol: phospholipid ratio were observed in the experiment. Once the experiment had indicated that physical activity really affected both the serum lipid levels and atherogenesis, the effect of physical activity on body weight and on the activity of the thyroid was taken into account in the hope of gaining an insight into the *modus operandi* of physical activity. The effect on body weight attracted attention because according to *Mann*'s group physical activity affects the serum lipid levels by preventing a positive caloric balance, i.e. by preventing weight gain. Attention was directed to thyroid activity chiefly because physical activity was found in this investigation to have a very similar effect to that exerted in some earlier investigations by the thyroid hormone on the serum cholesterol and atherogenesis.

Thus the main problems of the present study are:

(1) What is the effect of physical activity on the total serum cholesterol and phospholipid levels, on the total cholesterol: phospholipid ratio, and on the cholesterol content of the alpha and beta lipoproteins in cockerels on a commercial poultry diet and in cholesterol-fed cockerels?

(2) What is the effect of physical activity on the incidence and severity of spontaneus and cholesterol-induced atherosclerosis in cockerels?

(3) What is the effect of the different degrees of physical activity used in the present study on the weight of the birds?

(4) What is the effect of the different degrees of physical activity used in the present study on the activity of the thyroid glands of the birds?

Material

The experimental animals were White Leghorn cockerels which had received ordinary chick feed until the beginning of the experiment. At about seven weeks of age the cockerels were divided into four groups.

(1) *The active cholesterol-fed group.* Physical activity was unlimited and they were placed on a cholesterol diet. 35 cockerels were in this group at the end of the experiment.

(2) *The inactive cholesterol-fed group.* The physical activity was limited. The birds were fed the same cholesterol diet as those in the first group. 63 cockerels were in this group at the end of the experiment.

(3) *The active control group.* The physical activity was unlimited. The cockerels of this group were on the control diet. The group consisted of 21 cockerels at the end of the experiment.

(4) *The inactive control group.* The physical activity was limited. The diet was the same stock diet as in the third group. There were 26 cockerels at the end of the experiment.

Methods

Before starting the experiment the cockerels were weighed and a blood sample was taken from each for chemical analyses. The blood samples were taken from the alar vein, and before sampling the area was treated with vaseline to prevent hemolysis. The weighing and sampling took a week. As cockerels

grow older their weight and, as appeared in the present investigation, blood lipid levels change. However, the pre-experimental values of the weight and serum lipids were taken so much at random that the groups showed no differences as regards the initial values.

During the experiment, first two weeks excepted, blood samples were taken weekly from 1—10 individuals selected at random from each group. With a few exceptions, however, no individual gave more than one sample. The cockerels were weighed in connection with the sampling.

The experiment lasted 10—12 weeks. The cockerels were sacrificed during a fortnight. Hence for the first birds killed the experiment lasted 10 weeks, and for the last 12 weeks. The same proportion of the cockerels from each group was killed daily; hence the groups do not differ as regards the duration of the experiment. They were slaughtered by thrusting a broad, double-edged knife through the palate into the brain either via the mouth or, most usually, through the lower jaw. This causes copious bleeding. The samples for the blood analyses were taken from this blood.

At the end of the experiment 11 of the experimental animals turned out to be hens. They were not included in the material.

The experiment was complicated by *intestinal coccidiosis*. The disease appeared when the cockerels were about 6 weeks old, a week before the actual beginning of the experiment and immediately after the pre-experimental blood samples were taken. Thus far all the experimental animals had been together and all of them consequently had been equally exposed to the infection. The disease was diagnosed from autopsies and by microscopy at the State Veterinary Laboratory. All the affected animals and those in poor condition were killed immediately after the diagnosis was made, continuous and thorough disinfection measures were taken and sulphonamide therapy was started. Sulphonamide was administered by adding to the drinking water of the cockerels 0.2 % sulphadimidine sodium B.P. (120 c.c. of »Sulphamezatine» Sodium solution, 16 %, Imperial Chemical (Pharmaceuticals) Ltd. was added to 9 litres

of water). During the therapy this was the only drinking water available to the cockerels. Sulphonamide therapy was administered a week before the experiment, in the second experimental week and half a dose was given in the third experimental week. Of the cockerels killed because of the coccidiosis the majority were slaughtered a week before starting the experiment; a few cockerels in poor condition were killed at the beginning of the second experimental week. As the disease seemed to recur as late as the end of the fourth experimental week chlortetracyclin therapy (500 mg of Aureomycin Lederle was added to 8 litres of drinking water) was initiated and continued for the fifth and sixth week of the experiment. A total of 49 experimental animals was killed because of suspected infection. No signs of the disease were found at the autopsies made at the termination of the experiment.

Physical Activity

The most commonly used method, both in animal and human studies, to induce a difference in physical activity has been an artificial enhancement of activity in a part of the subjects. In the present study the opposite way was followed. The differene in physical activity was brought about by artificially limiting the activity of the cockerels of the inactive groups. These cockerels were confined in small cages (breadth 30 cm, length 30 cm and height 35 cm). The other cockerels, the first and third groups, were allowed to move freely in large pens. An endeavour was made to increase the activity of the cockerels in pens by building a dividing wall across the run, about 1 m high, and placing the food on one side of the wall and the drinking vessels on the other. Two planks led over the wall. The method employed produced a continuous difference in activity between the groups compared.

Confinement in small cages does not seem to be particularly detrimental to chickens. According to »Statens Husdjurförsök», hens kept in small cages even lay eggs better than hens free in

large enclosures but eat less and do not differ in weight from the free hens *(Olsson* 1954). Because both the cages and the pens were in the same large room, light, temperature, etc. conditions were the same. Thus the only important difference in the conditions of the active and inactive groups seems to have been the difference in physical activity.

The Diets

The cholesterol diet contained 1.5 per cent of cholesterol (Merck). The cholesterol was homogeneously mixed into commercial chick mash (Kasvatus-Tipu, Hankkija) in the same large feed mixer as is used for making the commercial mash.

According to information supplied by the manufacturer (Hankkija), the composition of the commercial mash was as follows:

Maize meal	25.500 %
Wheat meal	10.000 »
Oat meal	17.000 »
Wheat bran	17.000 »
Soya meal	3.000 »
Linseed meal	4.000 »
Sunflower meal	4.000 »
Bone meal	5.000 »
Fish meal	7.000 »
Hay meal	4.000 »
»Hen-Vitan», A + B + D vitamin concentrate *	1.000 »
»Deltafor D 3» **	0.200 »
Fodderlime	1.459 »
Coal meal	0.400 »
Sodium chloride	0.440 »
Copper sulphate	0.001 »
Total	100.000 %

* »Hen-Vitan» contains 900 international units per gram of vitamin A, 150 international units per gram of vitamin D_3 and 0.03 per cent of vitamin B_2.

** »Deltafor D 3» contains 200 international units per gram of vitamin D_3.

The commercial Mash consisted of 4 per cent of crude fat and 20 per cent of crude protein.

The commercial mash was used as the stock diet.

The cockerels got mashes and water *ad libitum*, plus small amounts of shell, but nothing else.

Chemical Methods

Serum was used for the analyses. The analyses were made within five days of taking the sample. The samples were stored in a refrigerator. Total cholesterol and phospholipid were determined from each initial, intermediate and final sample. Owing to the limited capacity of the paper electrophoresis apparatus it was not possible to fractionate lipoproteins for all initial and final samples, but almost all intermediate samples could be fractionated.

To establish the reliability of the methods, duplicate analyses of total cholesterol and phospholipid were made from all the intermediate samples; the same applied to a few lipoprotein determinations.

Table 1.

Standard Errors of Single Chemical Determinations

	Number of Duplicate Analyses	s_M
Total Cholesterol	86	10.3 mg/100 ml
Total Phospholipid	80	16.5 mg/100 ml
Beta Lipoprotein Cholesterol (per cent of Total)	12	1.3 per cent

The determination of total cholesterol and phospholipid

0.5 ml of serum was added slowly dropwise to 15 ml of alcohol-ether solution (3: 1), the mixture was shaken and kept in a boiling water bath until it had boiled for 1 minute. The flasks were cooled and allowed to stand overnight. On the following day the volume was adjusted to 20 ml and

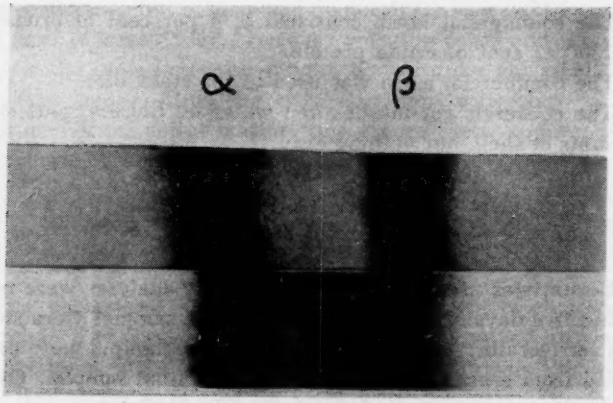

Fig. 1. Separation of alpha and beta lipoproteins. Lipidogram (above) and corresponding proteidogram.

the sediment centrifuged off. 5 ml aliquots were used for analyses of cholesterol and phospholipid. For cholesterol determination the mixture was evaporated in a water bath, the residue dissolved in hot chloroform. The chloroform extract was adjusted in a special tube to 5 ml; 2 ml of acetic anhydride-concentrated sulphuric acid mixture (20: 1) was added to the tube and mixed well. The tube was allowed to stand at 37°C for 30 minutes whereupon a colorimetric reading was taken at 625 mμ with a Coleman spectrophotometer. Lipid phosphorus was determined after ashing the ethanol-ether mixture according to the method of *Fiske* and *Subbarow* (1925) at 660 mμ. The factor 25 was used to convert lipid phosphorus to phospholipid.

Fractionation of lipoproteins by paper electrophoresis

The apparatus used corresponds in the main to the type introduced by *Durrum* (1951). An apparatus exactly similar to that employed in the present investigation was used by *Nikkilä* (1953). The paper strips rest upon a glass support frame to form a wide angle opening downwards. The electrode vessels are divided along their length into two parts by a dividing wall near the base of which there is, however, a row of holes connecting up the different parts of the vessel. On one side of the partition is a platinum wire electrode drawn through the whole vessel. The ends

of the strips of paper hang down from the support frame into the other side of the partition. The vessels and the support frame are enclosed in a chamber which can be removed as required. The vessels and the enclosed chamber are made of perspex. The apparatus accommodates 4 paper strips 11 cm wide at a time. The characteristic features of the apparatus are that it has a large humid gas chamber and that the fractionating takes place on paper sloping gently downward.

Schleicher et Schüll 2043 B electrophoresis paper was used, in strips of 36 × 11 cm. Barbital buffer, pH 8.6, (*Michaelis* 1931) was employed. On each strip 0.1 or 0.2 ml of serum was pipetted. When 4 strips were used and the voltage was 300 V the current was about 40 mA at the beginning of the run and at its end, after 6—8 hours, about 50—60 mA. The run completed, the paper strips were dried at room temperature. Orientating localisation was made in ultraviolet light in which the albumin zone fluoresced. After this, a strip about 2 cm wide was cut off the edge of the paper and stained with Sudan black according to the method of *Swahn* (1953) (Fig. 1). With the aid of this strip the places corresponding to alpha and beta lipoproteins were cut off the rest of the paper and lipids were extracted in alcohol-ether mixture from the pieces. Cholesterol was determined from the extract by the method reported above. The results of the analyses showed the relative cholesterol content of the alpha and beta lipoprotein fractions. With the total serum cholesterol level known it was possible to calculate the actual content of the alpha and beta lipoproteins.

Grading of Atherosclerotic Lesions

The cockerels were grouped on the basis of the lesions observed in macroscopic inspection into five classes which are taken to represent the different degrees of severity of atherosclerosis.

Examinations and grading of the vessels was performed as follows: A blind autopsy was made. On sacrificing a cockerel the original number plate was removed from its leg and a new one attached, enclosed in an envelope. Every day cockerels from each group were killed and autopsied. The aorta, the brachiocephalic and iliac arteries were examined in situ. The lesions observed were recorded graphically on special forms. Each lesion observed was entered on a diagram of the vascular

system at the spot and on more or less the scale of its actual occurrence in the vessels. Different colours were employed to indicate the nature (colour, elevation, roughness) of the lesions. On having completed the autopsy, the number in its sealed envelope was detached from the leg of the cockerel and enclosed with the form. The final classification took place solely on the basis of the forms and with the author still unaware of the identity of the bird.

The criteria for the classification according to macroscopic inspection were as follows:

Grade 0: Normal vessel (Fig. 2).

Grade 1: Slight yellowness. No other changes. Slight yellowness refers to yellowness occurring over a small area and as a rule weak in intensity. The surface of the vessel seems smooth. Changes of this type were found only in the elastic vessels, consequently not in the region of the abdominal aorta. Weak intimal and subintimal lipid accumulation was observed histologically in these areas (Fig. 3 and 4).

Grade 2: Marked yellowness and/or 1—3 minor lesions. Marked yellowness in elastic vessels indicates yellowness occurring over an extensive area or in several places. At such sites the intima has an uneven, granular appearance. Histologically there proved to be fairly pronounced lipid infiltration in the intima and especially in the subintimal tissue and adjacent media (Fig. 5). Regarded as marked yellowness in the muscular vessels were two types of lesions which were not elevated above their surroundings: (1) small yellow patches usually located on the anterior wall of the abdominal arch, quite proximal to the iliac arteries or in the orifices of the aortic branches, and (2) small, intensely yellow, rough, transverse lesions, sometimes found on the posterior wall of the abdominal aorta.

In elastic vessels the definition minor lesion is given to about 2—3 mm long and 1—1½ mm wide lesions which are clearly demarcated from their surroundings, elevated, yellow, often with a fairly smooth surface, generally situated longitudinally

Fig. 2. Normal thoracic aorta. Frozen section, Sudan III and hematoxylin stain, X 55.

Fig. 3. Slight yellowness. Thoracic aorta showing intimal thickening and minimal lipid infiltration beneath the intima in the adjacent media. Frozen section, Sudan III and hematoxylin stain, X 55.

Fig. 4. Slight yellowness. Thoracic aorta showing lipid infiltration of the intima and adjacent media. Frozen section, Sudan and hematoxylin stain, X 55.

Fig. 5. Marked yellowness. Brachiocephalic artery showing extensive lipid deposition in the intima and adjacent media. Frozen section, Sudan III and hematoxylin stain, X 90.

to the vessel (Fig. 6). In the histological specimen they proved to be lesions in which there was abundant lipid accumulation in the intima, the subintimal tissue and also in the media at the site of the lesion and in addition, intimal and subintimal proliferative changes (Fig. 8). In muscular vessels minor lesions refer to intensely yellow lesions of the same size as above, clearly elevated from their surroundings and found on histological examination to be similar to major lesions but smaller in size. Their localisation was also similar to that of major lesions in muscular vessels. In addition to these changes regarded as criteria, there occurred in the elastic vessels also the yellowness mentioned in the foregoing whereas quite isolated lesions often occurred in the muscular vessels.

Grade 3: 1—2 major lesions or 4 or more minor lesions. In elastic vessels major lesions refer to elevated, coarse-surfaced, yellow lesions over 3 mm long and over 2 mm wide, generally longitudinal to the vessel. Unevenness often appears in them as longitudinal folds (Fig. 7). Histologically, such lesions displayed besides lipid infiltration in the intima and the subintimal part also considerable lipid infiltration in the media, frequently spreading over a more extensive area than the intimal lesion (Fig. 9 and 10). In the muscular arteries lesions classified as major lesions are large, considerably elevated, yellow and uneven. In many cases the lumen of especially the iliac artery was greatly narrowed by the lesion. Lesions of this type are located most usually in the ventral wall of the abdominal aorta in the inter-renal region and in the most proximal part of the left iliac artery; they were found but rarely in the corresponding place on the right side. Histologically such lesions displayed a very marked lipid deposit in the subintimal layer; the media below it proved to be atrophic and sometimes quite absent (Fig. 11 and 12). In addition to these changes regarded as criteria of this class other changes, yellowness and minor lesions occurred in both elastic and muscular vessels.

Grade 4: 3 or more major lesions. In addition to these there were also other atheromatous changes.

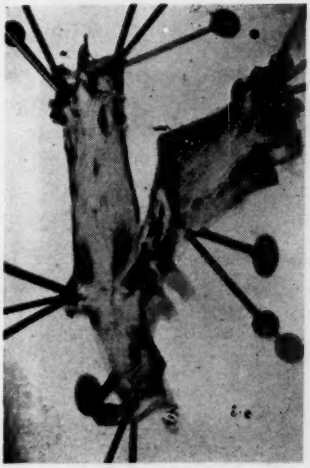

Fig. 6. Minor lesions in the right brachiocephalic artery. Gross staining, Sudan IV, about actual size.

Fig. 7. Major lesions in the thoracic aorta and brachiocephalic arteries. Gross staining, Sudan IV, about actual size.

Fig. 8. Minor lesion in the brachiocephalic artery. Local thickening and marked lipid deposition in the intima. Local medial involvement, though the media is otherwise lipid-free. Frozen section, Sudan III and hematoxylin stain, X 125.

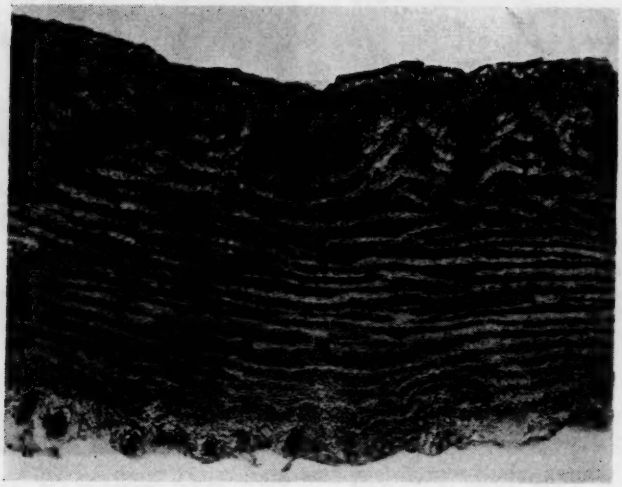

Fig. 9. Major lesion in the thoracic aorta. Extensive lipid deposition in the intima and media up to the outer portion of the media. Frozen section, Sudan III and hematoxylin stain, X 90.

The elastic arteries (the brachiocephalic arteries and the thoracic aorta), the muscular arteries (the abdominal aorta and the iliac arteries) and the whole vascular system examined (the aorta, the brachiocephalic and iliac arteries) were classified separately.

Four things were regarded as important in making up the classification. These were (1) that the classification should be descriptive so that the reader would know the kind of atheromatous changes represented by each class, (2) that the lesions used as criteria should be so simple that no marked errors could arise in their interpretation, (3) that the most probable errors in classification should be known and (4) that it should be possible to draw some quantitative conclusions about the classification. The gross staining method was employed in making the classification and checking its reliability. 65 vessels, including the elastic aorta and brachiocephalic arteries were set

Fig. 10. Major lesion in the thoracic aorta. Also other atheromatous changes. Frozen section, Sudan III and hematoxylin stain, X 15.

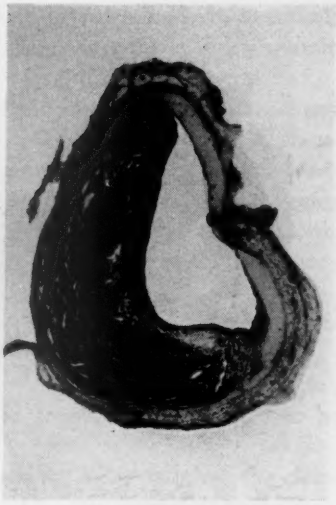

Fig. 11. Major lesion in the iliac artery. Lipid material abundant in the plaque, the media atrophic. Frozen section, Sudan III and hematoxylin stain, X 18.

aside and stained with Sudan IV. Before this, the changes observed without staining in the usual macroscopic examination were recorded on the form. A blind re-examination was made of the vessels stained with Sudan IV, the observations being recorded by the system used in the ordinary macroscopic examination. Hence two illustrations were available of each vessel, one based on ordinary examination and the other based on an inspection made after lipid staining. These pictures were compared. The comparison showed that in about half of the cases where marked yellowness was observed by ordinary examination a few minor lesions were revealed in addition after staining. This indicates that a vascular area interpreted as markedly yellow by ordinary examination often also displays an occasional minor lesion which may not but can just as well escape attention. Grade 2 is consequently considered to include vessels

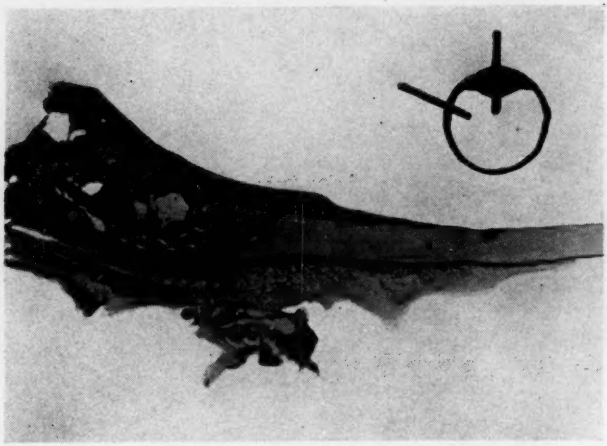

Fig. 12. Major lesion in the abdominal aorta. Frozen section, Sudan III and hematoxylin stain, X 25.

with marked yellowness and/or a few minor lesions. Errors were likewise observed to have originated in the interpretation of adjacent minor lesions and one or two major lesions. It was noticed after staining that what was a major lesion had been interpreted in the ordinary examination as a number of minor lesions crowded together, or vice versa. Hence the observation of 1—2 major lesions or over 4 minor lesions was regarded as the same thing for classification in Grade 3, and both or either of them were accepted as the criterion. It seems likely, judging by the gross staining inspection and histological specimens, that major lesions are formed through the confluence of minor lesions.

The observations based on ordinary examination and those made after staining were compared only in the elastic vessels (thoracic aorta and brachiocephalic arteries). The changes noted by the simple examination were much more distinct in the area of the muscular vessels (abdominal aorta and iliac

arteries) than in the elastic vessels, which makes it unlikely that markedly erroneous observations were made in the muscular arteries.

Comparison of the findings of the two methods of inspection also helps to assess the accuracy of the classification. The observations by both methods entered on the forms were independently analysed according to the classification made. Assuming that the gross staining method is more reliable, which it obviously is, errors occurred in observations based on ordinary inspection as follows:

— 27 cases were classified as grade 0. After staining, 23 of them were confirmed but 4 were judged to be grade 1;
— 6 cases were placed in grade 1. After staining, 2 were confirmed, 1 was placed in grade 2, and 3 were judged to be grade 0;
— 10 cases were classified as grade 2. After staining, the classification of 8 was confirmed and 2 were moved to grade 3;
— 7 cases were placed in grade 3. All were similarly classified after staining;
— 15 cases were placed in grade 4. All were similarly classified after staining.

This comparison shows that classification errors occur in grades 0 and 1. Of the cases considered to be normal upon ordinary inspection (grade 0), the staining method showed a slight lipid deposit on the intima in every eighth case (grade 1); on the other hand, in the six cases where simple inspection detected slight yellowness (grade 1), three, i.e. a half, proved normal by the staining method. No errors, however, were found in the interpretation of the most marked changes. Even for the cases checked by means of gross staining the final classification was based on the findings in an ordinary inspection.

Although the classification employed here is of a qualitative and descriptive nature it gives some quantitive evaluation of atherosclerosis, i.e. the different grades represent the different degrees of severity of atherosclerosis. Grade 0 stands for a normal vessel; grades 1—4 represent a vessel affected by atherosclerosis of rising degrees of severity. That the higher grades

represent atherosclerosis of a more severe degree than the lower grades is based on the following points:

(1) *Differences in the structure of the lesions.* The morphological patterns of the lesions used as classification criteria, yellowness, marked yellowness, minor lesions, major lesions, represent in this order more advanced atheromatous changes. This is in accordance with the view of *Chaikoff* et al. (1948) and that of the Michael Reese group (*Katz* and *Stamler* 1953).

(2) *Simultaneous presence of other types of lesion.* Lesions regarded as criteria of lower grades were also observed practically always in cases placed in a higher grade. Hence it does seem that lesions constituting criteria for the lower grades are intermediate phases in the growing severity of the atheroma. This view is also supported by the histological structure of the lesions and by the observations made by gross staining.

(3) *Frequency of the lesions.* For instance, more minor lesions are required for inclusion in grade 3 than for grade 2, and more major lesions must be present to qualify for placement in grade 4 than for grade 3.

Although the severity of atherosclerosis obviously increases on moving to higher grades, there are no grounds for assuming that the classification was linear.

In practice, this grading method resembles the method used by the Michael Reese group (*Horlick* and *Katz* 1949, *Katz* and *Stamler* 1953), but the differentiation of the degree of severity of the atherosclerosis deviates from the latter in principle. In the present method the vessels are classified in principle according to qualitative and descriptive findings into grades which obviously represent the different severity degrees of the atherosclerosis, but no claim is made for the linearity of the classification. The system of the Michael Reese group is no classification system but purports to be a measuring method, and the severity of atherosclerosis in the vessels is denoted by a number which is employed as a measurement. There is, however, no proof of the linearity of the Michael Reese group's grading system and therefore the number obtained cannot be used as

a measurement e.g. for the calculation of means or the statistical treatment of the results. However, there is no reason to doubt that the method, used with experience, gives a reliable picture of the condition of the vessel, but a person not familiar with the method will find it difficult to adopt the standardised technique required for the assessment of the changes.

The method employed in the present investigation is perhaps more approximative than the Michael Reese group's method but it has its own advantages. As the grades are qualitative the reader knows from the classification the kind of changes that the vessel has undergone. In these circumstances he is able himself to reflect on the possible difference in severity between the grades. As the method employed in the present investigation is a classification based on distinctly agreed criteria, the results obtained by this method can be treated statistically without any theoretical difficulties just as in any other case concerning frequency distribution into classes.

Estimation of Thyroid Activity

The histological structure of the thyroid is illustrative of the activity of the gland. An inactive thyroid gland is characterised by an abundance of colloid and a low epithelium. In an active thyroid the relative colloid volume is lower and the epithelium higher. The most useful quantitative histological methods employed in the estimation of thyroid activity are based in fact on the measurement of the relative volume of colloid or epithelium (*Uotila* and *Kannas* 1952) or on the measurement of the average height of the epithelial cells (*Rawson* and *Starr* 1938, *Rawson* and *Salter* 1940). Results of investigations where the effect of thyrotropic hormone on the relative volume of colloid or epithelium has been observed and those obtained in studies in which the above-mentioned histological characteristics have been compared with some other independent method of measuring glandular activity (*Uotila* and *Friedgood* 1943, *Tala* 1952, *Lamberg* 1953, *Wahlberg* 1955) show that the methods

based on the measurement of relative volume of colloid or epithelium are valid, reflecting glandular activity at least in experimental conditions. The results of *Tala*'s investigation (1952) also showed that it is equally good to use either the relative colloid volume or epithelium volume as an indicator of glandular activity.

In the present study the relative colloid volume of the thyroid was estimated by the systematic point sampling method (*Chalkley* 1943, *Eränkö* and *Kihlberg* 1955).

As far as possible, the sample for the measurement was always cut from the same place in the thyroid. The section from the middle of the gland was selected as the sample that could be localised most precisely and that best represented the gland as a whole. Several longitudinal sections were taken serially from the part with the largest cross-section of the fixed, slightly oval gland. They were mounted on a slide and stained with hematoxylin-eosin. Of the sections the largest in size was selected for the measurements and it consequently corresponded most probably to the desired point in the gland. Only this one sample was measured. A sample was taken without any selection from one of the two thyroids.

The relative volume of colloid was estimated in the following way. The image of the specimen was projected with a microprojector on a paper ruled into squares. The paper was fixed so that the transversal and longitudinal lines drawn coincided with the direction of movement of the slide holder of the projector. The 10 transversal and vertical lines constituted 100 intersection points. The whole sample was examined by moving the slide systematically along or across a distance of 10 squares at a time. In each position of the slide the number of blank intersection points (points outside the sample, on the connective tissue capsule, or on the large blood vessels) and the number of points on the epithelium and stroma were counted. The colloid points were obtained by deducting the blanks and the epithelium and stroma points from one hundred. The score thus obtained for each sample for the different positions of the slide were added up. An average of 5—6 positions was required per sample and the

average of intersection points coinciding with actual glandular tissue was 300—400. Because the portion of the stroma was found to be so small that it could not be differentiated with sufficient accuracy from epithelium, the points for these two tissues were not reported separately. This is in accordance with the observations and, in this respect, with the methods of *Lamberg* (1953) and *Wahlberg* (1955).

On autopsy the thyroids were weighed with a torsion balance. By using the value for the relative colloid volume obtained by the above-mentioned method, the amount of colloid, and that of the non-colloid portion of the glands, was calculated in mg. The weight of colloid and other tissues obtained is obviously somewhat approximative since in the periphery the follicles are usually larger than in the inner parts of the gland and because of possible differences in special weights of different tissue components. In spite of this, the values obtained from different experimental animals are comparable and may give more information about the glands than the volume percentages only, since by this means the weight of the glands is also taken into account.

Statistical Methods

In all the numerical analyses common statistical methods were applied. These are described in detail in several text-books (e.g. *Eränkö* and *Kihlberg* 1955).

When pre-experimental and final observations were compared it was possible, as far as the values of total cholesterol, phospholipid, the cholesterol: phospholipid ratio and body weight were concerned, to analyse the effect of the experiment on the basis of longitudinal observations. In these cases the difference between final and pre-experimental determinations was computed for each animal separately, whereafter the statistical significance of the average difference was determined by applying »*Student's*» t-test.

Lipoprotein determinations both at the beginning and at the end of the experiment were not carried out for all the experi-

mental animals but only from random samples representing each experimental group. In these cases the classical t-test was applied, often in the form of variance analysis. These analyses, of course, were not based on the observed individual changes but on the absolute observations before the experiment and at the end of it.

In analysing differences between the experimental groups during the experiment, variance analysis was applied separately within each intermediate week of the experiment. By eliminating the variation between the weeks, a pooled analysis was constructed; this analysis consequently revealed all the pooled information concerning differences between the groups during the experiment. In these analyses only the first eight experimental weeks were taken into account.

A similar pooled analysis was constructed in order to reveal the differences between the different grades of atherosclerosis.

In general, variance analysis was widely applied in the present work. In order to demonstrate the type of analysis applied in each separate case, the analysis tables are shown in abbreviated form, indicating the breakdown of the total variability, the number of degrees of freedom allocated for different sources of variation, and the results of the variance ratio or v^2 tests.

In reporting the results of statistical tests of significance, the probability P is shown. This quantity indicates the likelihood that the observed difference (or differences) was (were) due to chance only. If the probability lay above the conventional limit 0.05, its numerical value was replaced by two dots (..), indicating that the observed difference likely is due to chance only and that in these circumstances the exact value of P is of minor interest.

The methodical errors of the chemical determinations are indicated by means of the standard error of a single determination. This characteristic was calculated on the basis of double determinations, applying common formulas given e.g. by *Eränkö* and *Kihlberg* (1955).

Results

Serum Total Cholesterol

(Tables 2 and 3, Fig. 13)

In the control groups the cholesterol values rose from 106—113 mg/100 ml to 130—149 mg/100 ml, expressed as the range of means of each group, during the experiment. The increase was significant. No difference appeared between the active and inactive control groups. In the cholesterol-fed groups the cholesterol values rose markedly, and the difference against the control groups was clear. The cholesterol values reached their peak, an average of 367—501 mg/100 ml, in the fourth and fifth week of the experiment. This seemed to be followed by a drop until, at the end of the experiment, from the eight week on, there was again a rising tendency. Up to the beginning of the eighth week of the experiment the cholesterol values of the active cholesterol-fed group remained lower than those of the inactive cholesterol-fed group. After it the difference disappeared. Pooled analysis showed that the difference between the active and inactive cholesterol-fed groups, tested up to the beginning of the eighth week was significant at the level $P = 0.001$. As regards the final values, there was no difference between the two groups.

Comment. The pre-experimental values and the values of the control groups obtained in the present investigation concurred in broad outline with those observed in previous investigations (*Chaikoff* et al. 1948, *Katz* and *Stamler* 1953, *Nikkilä* and *Ollila* 1957).

No marked change in the plasma cholesterol level with age was found in earlier investigations of chicken on a normal diet (*Rodbard* et al. 1950, *Katz* and *Stamler* 1953, p. 253). A slight but nevertheless significant rise in the cholesterol level, observed in the present investigation, in cockerels kept on the control diet may be explained by the fact that in the poultry

Table 2.

Total Cholesterol (mg/100 ml) Before, During and at the End of the Experiment

Time of Sampling	*Active Chol.-Fed Group*	*Inactive Chol.-Fed Group*	*Active Control Group*	*Inactive Control Group*
Pre-Experimental				
Number	41	75	32	38
Mean	103	106	106	113
S.D.	7.2	11.0	7.8	14.1
3rd Week				
Number	4	6	4	4
Mean	137	252	106	109
4th Week				
Number	4	7	3	4
Mean	367	470	97	93
5th Week				
Number	10	9	—	—
Mean	179	501		
6th Week				
Number	4	7	4	3
Mean	183	233	91	97
7th Week				
Number	4	8	3	3
Mean	262	403	94	120
8th Week				
Number	3	7	2	3
Mean	195	248	97	120
9th Week				
Number	4	5	3	3
Mean	448	363	126	191
At the End				
Number	34	55	21	24
Mean	397	409	130	149

yard where the cockerels lived up to the age of about five weeks the diet was different from that used in the experiment. Thus, on the basis of the present study, no conclusion can be drawn about the effect of ageing *per se* on serum lipids in cockerels.

Table 3.

Testing the Differences in Total Cholesterol

a. *The Increase of Total Cholesterol in Control Groups, t-Test:*

	Number of Observations	*Mean Increase (mg/100 ml)*	*Standard Error of the Mean*	*P*
Active Control Group	20	23.0	7.0	0.01
Inactive Control Group	23	15.1	4.0	0.001

b. *Difference between the Active and Inactive Chol.-Fed Groups during the Experiment up to the Beginning of the 8th Experimental Week, Pooled Variance Analysis:*

Source of Variation	*Degrees of Freedom*	*P*
Between the Groups	6	0.001
Within — » —	61	•
Total	67	•

Average Residual Standard Deviation 112 mg/100 ml

Attention is attracted by the rapid rise of the cholesterol level in the cholesterol-fed groups and the occurrence of peak values as early as in the fourth and fifth week of the experiment, as well as by the subsequent drop in the total serum cholesterol. The possible role in this phenomenon of coccidiosis and its treatment will be discussed later (page 62). Most probably the phenomenon is that observed by *Rodbard* et al. (*Rodbard* et al. 1950, *Katz* and *Stamler* 1953, p. 251), viz. that in the cholesterol-fed chicken cholesterolemia reached its peak when the chickens were 8—10 weeks old, regardless of the different duration of cholesterol feeding; one series of animals had been on cholesterol diet from the first day of age, the others from the sixth and seventh week of age on. After the peak values the authors observed a subsequent slight decline in cholesterol level. In the birds on a normal diet this phenomenon was not observed. The investigators in question assumed that this fluctuation in the cholesterol level was due to some endogenous factor.

Serum Total Phospholipid

(Tables 4 and 5, Fig. 13)

Cockerels kept on the control diet registered a slight increase, from mean values of 305—309 mg/100 ml to those of 310—314 mg/100 ml, in the total phospholipid level during the

Table 4.

Total Phospholipid (mg/100 ml) Before, During and at the End of the Experiment

Time of Sampling	*Active Chol.-Fed Group*	*Inactive Chol.-Fed Group*	*Active Control Group*	*Inactive Control Group*
Pre-Experimental				
Number	*41*	*75*	*32*	*38*
Mean	302	302	309	305
S.D.	35.6	31.1	24.4	31.9
3rd Week				
Number	*4*	*6*	*4*	*4*
Mean	338	379	294	339
4th Week				
Number	*4*	*7*	*3*	*4*
Mean	432	509	325	309
5th Week				
Number	*10*	*9*	—	—
Mean	322	394		
6th Week				
Number	*4*	*7*	*4*	*3*
Mean	448	488	304	323
7th Week				
Number	*4*	*8*	*3*	*3*
Mean	326	353	319	342
8th Week				
Number	*3*	*7*	*2*	*3*
Mean	350	347	339	352
9th Week				
Number	*4*	*5*	*3*	*3*
Mean	334	335	359	382
At the End				
Number	*34*	*55*	*21*	*24*
Mean	363	367	314	310

Table 5.

Testing the Differences in Total Phospholipid

a. *Differences between the Pre-Experimental and Final Values, t-Test:*

	Number of Observations	*Mean Increase (mg/100 ml)*	*Standard Error of the Mean*	*P*
Active Chol.-Fed Group	31	58.5	7.9	0.001
Inactive Chol.-Fed Group	54	64.9	7.0	0.001
Active Control Group	20	27.4	6.3	0.001
Inactive Control Group	23	13.5	8.0	..

b. *Difference between the Active and Inactive Chol.-Fed Groups during the Experiment up to the Beginning of the 8th Experimental Week, Pooled Variance Analysis:*

Source of Variation	*Degrees of Freedom*	*P*
Between the Groups	6	0.05
Within — » —	61	.
Total	67	.

Average Residual Standard Deviation 56 mg/100 ml

c. *Effect of Cholesterol Feeding and Physical Activity on the Differences between the Pre-Experimental and Final Values, Variance Analysis:*

Source of Variation	*Degrees of Freedom*	*P*
Cholesterol Feeding	1	0.001
Physical Activity in Chol.-Fed Groups	1	..
— » — in Control Groups ..	1	..
— » — pooled	2	..
Residual	124	.
Total	127	.

experiment. In the active control group the increase was significant at the level P = 0.001, but in the inactive control group insignificant. No difference was observed between the active and inactive control groups. In the cholesterol-fed groups phospholipid values increased more clearly; the peak values, 432—509 mg/100 ml, coincided with the fourth week of the experiment and were followed by a falling tendency from the sixth week on. The values of the active group kept on an average below the values of the inactive group until the beginning of

the eight week when the difference disappeared. The difference when tested up to the beginning of the eighth week was significant at the level P = 0.05. The final values of the active and inactive cholesterol-fed groups did not differ, but the values of the cholesterol-fed groups were significantly higher, P = 0.001, than the values of the control groups.

Comment. The normal values of the material (the pre-experimental values and those of the control birds), from 294 to 382 mg/100 ml, expressed as the range of means of different groups, are of the same magnitude as the values given by *Nikkilä* and *Ollila* (1957) for Finnish chicken of the same breed. *Chaikoff* et. al. (1948) reported distinctly lower values, 125—223 mg/100 ml, and according to the Michael Reese group (*Katz* and *Stamler* 1953) the normal values of lipid phosphorus were 7.5—7.8 mg/100 ml which, converted into phospholipids by the factor 25, makes 187.5—195 mg/100 ml. The difference between the values obtained in Finland and the United States probably admits no other explanation than that of racial differences between the experimental animals used.

The Ratio Cholesterol: Phospholipid

(Tables 6 and 7, Fig. 13)

A small rise in the ratio occurred in the control groups during the experiment, the increase in the active control group being significant at the level P = 0.02 and in the inactive control group at the level P = 0.001. The active and inactive control groups did not differ. There was a considerable increase in the ratio in the cholesterol-fed groups. It followed on the whole the fluctuation in the total cholesterol values reaching its peak, 0.83—1.25, in the fourth and fifth week. Then followed a minor drop and, at the end, again a rising tendency. The difference from the control groups was clear. Up to the beginning of the eighth week the values of the active cholesterol-fed group remained lower than those of the inactive cholesterol-fed group,

Table 6.

The Cholesterol:Phospholipid Ratio Before, During and at the End of the Experiment

Time of Sampling	*Active Chol.-Fed Group*	*Inactive Chol.-Fed Group*	*Active Control Group*	*Inactive Control Group*
Pre-Experimental				
Number	41	75	32	38
Mean	0.35	0.36	0.34	0.37
3rd Week				
Number	4	6	4	4
Mean	0.41	0.66	0.39	0.32
4th Week				
Number	4	7	3	4
Mean	0.83	0.90	0.30	0.30
5th Week				
Number	10	9	—	—
Mean	0.57	1.25		
6th Week				
Number	4	7	4	3
Mean	0.39	0.47	0.30	0.30
7th Week				
Number	4	8	3	3
Mean	0.78	1.10	0.30	0.32
8th Week				
Number	3	7	2	3
Mean	0.56	0.70	0.29	0.34
9th Week				
Number	4	5	3	3
Mean	1.32	1.06	0.35	0.50
At the End				
Number	34	55	21	24
Mean	1.08	1.10	0.41	0.48

the difference being significant at the level $P = 0.001$. At the end of the experiment there was no difference between the active and inactive cholesterol-fed groups.

Table 7.

Testing the Differences in the Cholesterol:Phospholipid Ratio

a. *Differences between the Pre-Experimental and Final Values, t-Test:*

	Number of Observations	*Mean Increase*	*Standard Error of the Mean*	*P*
Active Chol.-Fed Group	31	0.752	0.064	0.001
Inactive Chol.-Fed Group	54	0.737	0.079	0.001
Active Control Group	20	0.058	0.021	0.02
Inactive Control Group	23	0.081	0.021	0.001

b. *Difference between the Active and Inactive Chol.-Fed Groups during the Experiment up to the Beginning of the 8th Experimental Week, Pooled Variance Analysis:*

Source of Variation	*Degrees of Freedom*	*P*
Between the Groups	6	0.001
Within — » —	61	.
Total	67	.

Average Residual Standard Deviation 0.26

c. *Effect of Cholesterol Feeding and Physical Activity on the Differences between the Pre-Experimental and Final Values, Variance Analysis:*

Source of Variation	*Degrees of Freedom*	*P*
Cholesterol Feeding	1	0.001
Physical Activity in Chol.-Fed Groups	1	..
— » — in Control Groups ..	1	..
— » — Pooled	2	..
Residual	124	.
Total	127	..

Comment. The normal values, 0.30—0.50, do not differ from the values derived by calculation from the data of *Nikkilä* and *Ollila* (1957). On the other hand, the computed ratio from the results obtained by *Chaikoff* et al. (1948) and by the Michael Reese group (*Katz* and *Stamler* 1953) is much higher owing to the considerably lower phospholipid values they obtained. The changes in the ratio cholesterol: phospholipid observed in the experiment were chiefly due to the changes in the cholesterol level.

Cholesterol in Alpha and Beta Lipoprotein Fractions

The quantity of cholesterol in beta lipoproteins appeared to be higher than in the alpha lipoprotein fraction (Table 8). In the pre-experimental samples and in those obtained from the control groups 55.7—64.5 per cent of the total serum cholesterol was in the beta lipoprotein fraction. In the control groups the cholesterol content of the lipoprotein fractions increased a little (Tables 10 and 12), the increase in both lipoprotein fractions being significant at the level $P = 0.01$ (Tables 11 and 13). On the other hand, no change was observed in the distribution of cholesterol between the alpha and beta lipoproteins (Tables 8 and 9). The active and inactive control groups did not differ from each other. In the cholesterol-fed groups the cholesterol content increased markedly during the experiment in both lipoprotein fractions (Tables 10 and 12, Fig. 13). In the beta lipoprotein fraction the increase was greater and followed closely the variations of the total serum cholesterol level (Fig. 13). The smaller, but in any case significant, rise of the cholesterol content of the alpha lipoprotein fraction did not follow so clearly the changes in the total serum cholesterol level. A marked increase in the alpha lipoprotein fraction was observed only from the fifth week of the experiment on. Up to the beginning of the eighth week of the experiment the cholesterol values of both lipoprotein fractions remained lower in the active than in the inactive cholesterol-fed group. Pooled analysis of the intermediate values showed that the difference was significant at the level $P = 0.001$ in both the lipoprotein fractions (Tables 11 and 13).

As already indicated by the cholesterol content of the alpha and beta lipoproteins, a change occurred in the cholesterol distribution during the experiment in the cholesterol-fed groups but not in the control groups (Tables 8 and 9). In the cholesterol-fed groups the percentage of total serum cholesterol in the beta lipoprotein fraction rose markedly and the difference from the control groups was obvious. The peak values, 79—89 per cent

Table 8.

Percentage of Total Cholesterol in Beta Lipoprotein Before, During and at the End of the Experiment

Pre-Experimental (from 18 Randomly Chosen Birds) 62.3

Time of Sampling	*Active Chol.-Fed Group*	*Inactive Chol.-Fed Group*	*Active Control Group*	*Inactive Control Group*
3rd Week				
Number	4	4	2	4
Mean	67.1	74.2	64.5	59.5
4th Week				
Number	4	6	3	3
Mean	79.3	89.0	60.3	56.4
5th Week				
Number	10	6	—	—
Mean	61.4	66.8		
6th Week				
Number	3	7	1	3
Mean	64.7	63.2	55.7	56.6
7th Week				
Number	4	4	2	2
Mean	61.9	69.4	58.4	8.1
8th Week				
Number	3	3	1	—
Mean	62.7	64.3	59.3	
9th Week				
Number	2	4	1	2
Mean	61.8	61.6	55.8	60.7
At the End				
Number	17	10	8	5
Mean	73.3	73.1	64.1	63.1

of total cholesterol in the beta lipoprotein fraction, were observed at the beginning of the fourth experimental week, followed by a distinct drop and, at the end, possibly a new tendency to rise. The values of the active cholesterol-fed group remained

Table 9.

Testing the Differences in Percentage of Total Cholesterol in Beta Lipoprotein

a. *Differences between the Pre-Experimental and Final Values and Effect of Cholesterol Feeding and Physical Activity on Final Values, Variance Analysis:*

Source of Variation	*Degrees of Freedom*	*P*
Between the Groups	4	0.001
Of which:		
Between Pre-Experimental and Final Values	1	0.001
— » — — » — — » — in Controls	1	..
In Final Values:		
Cholesterol Feeding	1	0.001
Physical Activity in Chol.-Fed Groups	1	..
— » — in Control Groups	1	..
— » — Pooled	2	..
Residual	53	.
Total	57	.

Average Residual Standard Deviation 5 per cent

b. *Differences between the Active and Inactive Chol.-Fed Groups during the Experiment up to the Beginning of the 8th Experimental Week, Pooled Variance Analysis:*

Source of Variation	*Degrees of Freedom*	*P*
Between the Groups	6	0.01
Within — » —	46	.
Total	52	.

Average Residual Standard Deviation 4.7 per cent

at a lower level than those of the inactive group. The difference, when tested up to the beginning of the eighth week of the experiment, was significant at the level P = 0.01.

Comment. Eder (1955) mentions having studied, by Cohn's method 10, the distribution of cholesterol and phospholipids in fractions IV + V + VI (corresponds mainly to alpha lipoproteins) and I + III (corresponds mainly to beta lipoproteins) in a single chick; he found a »pattern not greatly dissimilar to

Table 10.

Cholesterol Content (mg/100 ml) of Alpha Lipoprotein Before, During and at the End of the Experiment

Pre-Experimental (from 18 Randomly Chosen Birds) 42

Time of Sampling	*Active Chol.-Fed Group*	*Inactive Chol.-Fed Group*	*Active Control Group*	*Inactive Control Group*
3rd Week				
Number	4	4	2	4
Mean	45	69	34	44
4th Week				
Number	4	6	3	3
Mean	63	52	38	40
5th Week				
Number	10	6	—	—
Mean	68	164		
6th Week				
Number	3	7	1	3
Mean	70	83	41	42
7th Week				
Number	4	4	2	2
Mean	97	166	40	51
8th Week				
Number	3	3	1	—
Mean	72	73	41	
9th Week				
Number	2	4	1	2
Mean	182	166	63	89
At the End				
Number	17	10	8	5
Mean	103	113	50	47

man». The percentage distribution of cholesterol in normal cockerels observed in the present study is similar to the value obtained in healthy human subjects (*Nikkilä* 1953, *Eder* 1955, *Surgenor* 1955, *Jencks* et al. 1956). In atherosclerotic subjects

Table 11.

Testing the Differences in Cholesterol Content (mg/100 ml) of Alpha Lipoprotein

a. *Difference between the Pre-Experimental and Final Values in the Control Groups, Variance Analysis:*

Source of Variation	*Degrees of Freedom*	*P*
Between the Pre-Exp. and Final Values	1	0.01
Residual	22	•
Total	23	•

Average Residual Standard Deviation 7.3 mg/100 ml

b. *Difference between the Active and Inactive Chol.-Fed Groups during the Experiment up to the Beginning of the 8th Experimental Week, Pooled Variance Analysis:*

Source of Variation	*Degrees of Freedom*	*P*
Between the Groups	6	0.001
Within — » —	46	•
Total	52	•

Average Residual Standard Deviation 28 mg/100 ml

the values 76—87 per cent of total cholesterol in beta lipoproteins (*Nikkilä* 1953, *Eder* 1955, *Jencks* et al. 1956) have been obtained. These are higher than the corresponding values found in the cholesterol-fed cockerels at the end of the present experiment, but similar to the peak values during the experiment.

The observation that in the cholesterol-fed groups the absolute amount of cholesterol increased also in the alpha lipoprotein fraction does not agree with observations made on man. On the contrary, the cholesterol content of alpha lipoprotein in man has been found to fall in both atherosclerosis and hypercholesterolemic conditions (*Kunkel* and *Slater* 1952, *Nikkilä* 1953, *Eder* 1955, *Jencks* et al. 1956). Although the lipoprotein fractions of chicken and man perhaps resemble one another broadly speaking (*Lewis* et al. 1952, *Eder* 1955), fractionation by ultra-

Table 12.

Cholesterol Content (mg/100 ml) of Beta Lipoprotein Before, During and at the End of the Experiment

Pre-Experimental (from 18 Randomly Chosen Birds) 70

Time of Sampling	*Active Chol.-Fed Group*	*Inactive Chol.-Fed Group*	*Active Control Group*	*Inactive Control Group*
3rd Week				
Number	*4*	*4*	*2*	*4*
Mean	92	207	62	65
4th Week				
Nummer	*4*	*6*	*3*	*3*
Mean	305	458	58	52
5th Week				
Number	*10*	*6*	—	—
Mean	111	356		
6th Week				
Number	*3*	*7*	*1*	*3*
Mean	145	150	52	55
7th Week				
Number	*4*	*4*	*2*	*2*
Mean	166	395	56	73
8th Week				
Number	*3*	*3*	*1*	—
Mean	123	135	59	
9th Week				
Number	*2*	*4*	*1*	*2*
Mean	295	286	80	139
At the End				
Number	*17*	*10*	*8*	*5*
Mean	311	345	93	81

centrifuge has shown differences in the subfractions (*Lewis* et al. 1952). In these circumstances the alpha and beta lipoprotein fractions of chicken and man cannot be considered fully analogous nor can they be assumed to behave similarly.

Table 13.

Testing the Differences in Cholesterol Content (mg/100 ml) of Beta Lipoprotein

a. *Differences between the Pre-Experimental and Final Values in the Control Groups, Variance Analysis:*

Source of Variation	*Degrees of Freedom*	*P*
Between Pre-Experimental and Final Values	1	0.01
Residual	22	•
Total	23	•

Average Residual Standard Deviation 19.2 mg/100 ml

b. *Difference between the Active and Inactive Chol.-Fed. Groups during the Experiment up to the Beginning of the 8th Experimental Week, Pooled Variance Analysis:*

Source of Variation	*Degrees of Freedom*	*P*
Between the Groups	6	0.001
Within — » —	46	•
Total	52	•

Average Residual Standard Deviation 101 mg/100 ml

The Incidence and Severity of Atherosclerosis

In the control groups which were on the stock diet some atheromatous lesions were found in 8 of the 47 control birds (Table 14). In the elastic arteries the lesions were slight (Table 15), but in the muscular arteries there were also major lesions in three control cockerels (Table 16). The active and inactive control groups showed no mutual differences in the incidence and severity of the atheromatous lesions.

In the cholesterol-fed groups the difference between the active and inactive groups was extremely clear. In the classification taking into account all the arteries observed, the aorta and the brachiocephalic and iliac arteries, the vessels were found normal in 14 of the 35 birds of the active cholesterol-fed

Table 14.

Grading Based on All the Observed Arteries (the Aorta and the Brachiocephalic and Iliac Arteries)

	Active Chol.-Fed Group	*Inactive Chol.-Fed Group*	*Active Control Group*	*Inactive Control Group*
Grade 0	14	3	18	21
Grade 1	0	2	1	1
Grade 2	12	13	1	2
Grade 3	7	24	1	2
Grade 4	2	21	0	0
Total	35	63	21	26

Table 15.

Grading of the Elastic Arteries (the Thoracic Aorta and Brachiocephalic Arteries)

	Active Chol.-Fed Group	*Inactive Chol.-Fed Group*	*Active Control Group*	*Inactive Control Group*
Grade 0	23	4	19	24
Grade 1	3	7	1	1
Grade 2	6	23	1	1
Grade 3	2	12	0	0
Grade 4	1	17	0	0
Total	35	63	21	26

groups, and major lesions appeared only in 9 of them. But in the inactive cholesterol-fed group only 3 of 63 cockerels had normal arteries and 45 showed major lesions (Table 14). Confining the classification to the occurrence of atheromatous lesions in the elastic vessels, the thoracic aorta and brachioceph-

Table 16.

Grading of the Muscular Arteries (the Abdominal Aorta and the Iliac Arteries)

	Active Chol.-Fed Group	*Inactive Chol.-Fed Group*	*Active Control Group*	*Inactive Control Group*
Grade 0	16	14	20	23
Grade 1	0	0	0	0
Grade 2	11	14	0	1
Grade 3	8	31	1	2
Grade 4	0	4	0	0
Total	35	63	21	26

alic arteries, the difference was, if possible, still greater. The elastic arteries were normal in 23 of the 35 cockerels of the active group, but only in 4 of the 63 in the inactive group; major lesions were found in the elastic arteries in only 3 of the active but 29 of the inactive group (Table 15). In the muscular arteries, the abdominal aorta and the iliac arteries, the difference was smaller but still significant (Table 16).

Body Weight

(Tables 17 and 18)

The pre-experimental weights of the different groups did not differ. The activity had no effect on weight, but at the end of the experiment the weights of the cholesterol-fed birds appeared to be, on an average, a little lower than those of the control animals, the difference being significant at the level $P = 0.05$.

Table 17.

Body Weight (gr) of the Experimental Animals Before, During and at the End of the Experiment

Time of Weighing	*Active Chol.-Fed Group*	*Inactive Chol.-Fed Group*	*Active Control Group*	*Inactive Control Group*
Pre-Experimental				
Number	44	76	38	39
Mean	463	443	434	448
S.D.	80	86	92	66
3rd Week				
Number	4	6	4	4
Mean	675	896	780	784
4th Week				
Number	4	7	4	4
Mean	929	917	838	775
5th Week				
Number	10	9	—	—
Mean	932	1087		
6th Week				
Number	4	8	4	3
Mean	1094	1049	1158	932
7th Week				
Number	4	8	3	3
Mean	1225	1194	1257	1293
8th Week				
Number	3	7	2	3
Mean	1213	1129	1405	1197
9th Week				
Number	4	5	3	3
Mean	1363	1442	1347	1263
At the End				
Number	35	63	21	26
Mean	1563	1485	1651	1500

Table 18.

Testing the Differences in Body Weight

a. *Differences between the Groups in Pre-Experimental Weights, Variance Analysis:*

Source of Variation	*Degrees of Freedom*	*P*
Between the Groups	3	..
Residual	193	.
Total	196	.

b. *Effect of Cholesterol Feeding and Physical Activity on the Differences between the Pre-Experimental and Final Values, Variance Analysis:*

Source of Variation	*Degrees of Freedom*	*P*
Cholesterol Feeding	1	0.05
Physical Activity in Chol.-Fed Groups	1	..
— » — in Control Groups	1	..
— » — Pooled	2	..
Residual	135	.
Total	138	.

The standard errors of the mean increases in body weight were in the groups 32—45 gr.

The Thyroid Glands

(Tables 19 and 20)

The thyroid weights of the different experimental groups did not differ, but a comparison confined to cholesterol-fed groups only showed that the average weight of the inactive cholesterol-fed group was probably significantly ($P = 0.05$) higher than that of the active cholesterol-fed group. In the physically inactive groups the colloid percentage was higher in the cholesterol-fed than in the control group, the difference being significant at the level $P = 0.01$. In respect to cholesterol feeding the difference between the active groups was insignificant. The relative colloid volume in the inactive cholesterol-fed group was highly significantly ($P = 0.001$) greater than that in the active cholesterol-fed group. In the control groups no significant difference in respect to physical activity was found.

Table 19.

The Thyroid Glands

	Active Chol.-Fed Group	*Inactive Chol.-Fed Group*	*Active Control Group*	*Inactive Control Group*
Tyroid Weight (mg)				
Number	32	55	19	22
Mean	183	208	200	173
Relative Colloid Volume (per cent)				
Number	23	53	16	18
Mean	75	79	74	76
Colloid Content (mg)				
Number	23	53	16	18
Mean	138	172	151	126
Content of Epithelium and Stroma (mg)				
Number	23	53	16	18
Mean	43	43	51	39

Table 20.

Testing the Differences Concerning the Thyroids

a. *Differences in Thyroid Weight, Variance Analysis:*

Source of Variation	*Degrees of Freedom*	*P*
Between the Groups	3	..
Physical Activity in Chol.-Fed Groups	1	0.05
— » — in Control Groups	1	..
— » — Pooled	2	0.05
Residual	124	.
Total	127	.

Average Residual Standard Deviation 57 mg

b. *Effect of Cholesterol Feeding on Relative Colloid Volume, Variance Analysis:*

Source of Variation	*Degrees of Freedom*	*P*
Between the Active Groups	1	..
— » — the Inactive Groups	1	0.01
Other Differences Between the Groups	1	0.001
Residual	106	.
Total	109	.

c. *Effect of Physical Activity on Relative Colloid Volume, Variance Analysis:*

Source of Variation	*Degrees of Freedom*	*P*
Between the Chol.-Fed Groups	1	0.001
— » — the Control Groups	1	..
Other Differences Between the Groups	1	0.01
Residual	106	.
Total	109	.

Average Residual Standard Deviation of Relative Colloid Volume 4.3 per cent

d. *Effect of Cholesterol Feeding on Colloid Content (mg), Variance Analysis:*

Source of Variation	*Degrees of Freedom*	*P*
Between the Active Groups	1	..
— » — the Inactive Groups	1	0.01
Other Differences Between the Groups	1	..
Residual	100	.
Total	103	.

e. *Effect of Physical Activity on Colloid Content (mg), Variance Analysis:*

Source of Variation	*Degrees of Freedom*	*P*
Between the Chol.-Fed Groups	1	0.01
— » — the Control Groups	1	..
Other Differences Between the Groups	1	0.05
Residual	100	.
Total	103	.

Average Residual Standard Deviation of Colloid Content 49 mg

f. *Effect of Cholesterol Feeding on Content (mg) of Epithelium and Stroma, Variance Analysis:*

Source of Variation	*Degrees of Freedom*	*P*
Between the Active Groups	1	0.05
— » — the Inactive Groups	1	..
Other Differences Between the Groups	1	..
Residual	100	.
Total	103	.

g. *Effect of Physical Activity on Content (mg) of Epithelium and Stroma, Variance Analysis:*

Source of Variation	*Degrees of Freedom*	*P*
Between the Chol.-Fed Groups	1	..
— » — the Control Groups	1	0.01
Other Differences Between the Groups	1	..
Residual	100	.
Total	103	.

Average Residual Standard Deviation of Content of Epithelium and Stroma 11 mg

In further analysis of the results, where the changes in the amount, in mg, of colloid and of epithelium and stroma were observed, it appeared that the differences in the relative colloid volume were based chiefly on the changes in the total amount of colloid. The amount of colloid was significantly ($P = 0.01$) greater in the inactive cholesterol-fed group than in the inactive control group, but for epithelium and stroma there was no significant difference. Similarly, the amount of colloid in the inactive cholesterol-fed group was significantly ($P = 0.01$) greater than in the active cholesterol-fed group, but the amount of epithelium and stroma was, on an average, quite the same in these groups.

Comment. There is no doubt about the fact that a decreased relative colloid volume indicates metabolic activity, and increased relative colloid volume metabolic inactivity, also in the thyroid gland of chicken (*Lamberg* 1953, *Wahlberg* 1955). But thyroid activation has also been found to correlate positively with thyroid weight, also in chicken (*Keating* et al. 1945, *Lamberg* et al. 1955). In the present study differences in the relative and also in the absolute colloid contents were observed without concomitant changes in thyroid weights. Thus the interpretation of the results seems difficult. However, it has been shown that an increase in glandular weight is not inevitably correlated with glandular activity. *Wahlberg* (1955) did not observe, after the administration of thyrotrophic hormone, any significant changes in the thyroid weights of chicken although the amount of thyrotrophic hormone used in the test caused a clear metabolic activation of the thyroids, which appeared in an increased uptake of P_{32} and of I_{131} and was reflected significantly in the relative colloid volume. *Lamberg* et al. (1955) studied the effect of repeated stimulation with thyrotrophic hormone on the uptake of P_{32}, on the histological picture of the thyroid and on thyroid weight in young chicken. They found that although clear metabolic activation and a decrease in relative colloid volume appeared in a few hours, the weight gain was seen only after the third injection on the third day of the experiment. The studies mentioned indicate

that the relative colloid or epithelium volume reflects thyroid activity but not thyroid weight, at least not in all conditions. These studies also indicate that a stimulus which causes a metabolic activation and changes in the relative colloid volume may be different from that which influences thyroid weight.

Thus it seems justified to conclude that the changes observed in the present study in the relative and absolute amount of colloid reflect glandular activity.

Examination of the thyroids indicated that (1) thyroid activity in the inactive cholesterol-fed group was significantly lower than in the active cholesterol-fed group, but there was no significant difference in this respect in the control groups, and (2) the activity of the thyroids was significantly lower in the physically inactive cholesterol-fed group than in the inactive control group, but there was no significant difference as regards cholesterol feeding between the physically active groups.

These observations seem to indicate a correlation between the inactivation of the thyroids and both physical inactivity and cholesterol feeding. Obviously, however, there is a similar correlation between the inactivation of the thyroids and e.g. the severity of atherosclerosis and the serum lipid levels. In these circumstances it is impossible on the basis of the present study to decide precisely what factors influenced thyroid activity.

There is, however, some support for the idea that physical activity has an influence on thyroid activity; physical exercise at least is considered to increase thyroid function (*Hurxthal* and *Musulin* 1953, p. 307). *Brown—Grant* (1957) observed that forced immobilisation for 48 hours decreased thyroid activity in rabbits. He found an inhibition of the release of thyroidal radioiodine, which was associated with a fall in the $PB^{131}I$ below the predicted value. *Eränkö* and *Muittari* (1957) found that relative colloid volume, colloid content in mg and thyroid weight were greater in neurotic rats than in controls. Neurotisation was induced by braking a conditioned jumping reflex. The neurotic rats were more passive, less active and moved less than the control animals. In the above-mentioned investigations inactivation of the thyroids was obviously associated with physical inactiv-

ity, although it is impossible to claim that the inactivation was caused by physical inactivity; on the contrary, the authors regard it as a result of emotional stress *(Brown—Grant)* or neurotisation (*Eränkö* and *Muittari)*. On the other hand, there are reports which show that cholesterol feeding inhibits the effect of thyroid hormone (*Saegesser* 1933, *Marx* et al. 1948, *Winebrenner* and *Marx* 1949). The above-mentioned authors assume that cholesterol has a direct influence on thyroid hormone or on its metabolic effects and consequently not on the thyroid glands.

Correlation Between Serum Lipid Levels and Severity of Atherosclerosis

(Tables 21 and 22)

In the cholesterol-fed groups the total cholesterol, phospholipid and the ratio cholesterol: phospholipid obtained at the end of the experiment from cockerels with different grades of

Table 21.

Cerum Total Cholesterol (mg/100 ml) and the Cholesterol: Phospholipid Ratio at the End of the Experiment in Cockerels with Different Grades of Atherosclerosis

	Grade 0	*Grade 1*	*Grade 2*	*Grade 3*	*Grade 4*
Grading of Elastic Vessels:					
Total Cholesterol					
Number	*25*	*10*	*26*	*13*	*15*
Mean	355	277	435	454	474
Chol.:Phosphol. Ratio					
Number	*25*	*10*	*26*	*13*	*15*
Mean	1.00	0.81	1.22	1.21	1.20
Grading of Muscular Vessels:					
Total Cholesterol					
Number	*26*	—	*23*	*37*	*3*
Mean	376		370	426	627
Chol.:Phosphol. Ratio					
Number	*26*	—	*23*	*37*	*3*
Mean	1.06		1.05	1.13	1.47

Table 22.

Testing the Differences in Serum Lipid Values of Cockerels with Different Grades of Atherosclerosis, Pooled Variance Analysis:

a. *Elastic Vessels, Total Cholesterol:*

Source of Variation	*Degrees of Freedom*	*P*
Between the Grades	8	0.01
Residual	79	.
Total	87	.

Average Residual Standard Deviation 136 mg/ 100 ml

b. *Elastic Vessels, Cholesterol: Phospholipid Ratio:*

Source of Variation	*Degrees of Freedom*	*P*
Between the Grades	8	0.01
Residual	79	.
Total	87	.

Average Residual Standard Deviation 0.28

c. *Muscular Vessels, Total Cholesterol:*

Source of Variation	*Degrees of Freedom*	*P*
Between the Grades	5	0.05
Residual	82	.
Total	87	.

Average Residual Standard Deviation 144 mg/100 ml

d. *Muscular Vessels, Cholesterol: Phospholipid Ratio:*

Source of Variation	*Degrees of Freedom*	*P*
Between the Grades	5	..
Residual	82	.
Total	87	.

Average Residual Standard Deviation 0.29

atherosclerosis, were compared. The control groups were not considered because of the low incidence of atherosclerosis. The lipoproteins were determined only from a part of the experimental animals at the end of the test; consequently there was too little information on lipoproteins for this purpose.

When the classification concerning elastic arteries was used it was found that the total serum cholesterol values and the cholesterol: phospholipid ratios were, on an average, lowest in grade 0 and 1, and tended to rise in the higher grades of atherosclerosis. The total cholesterol level and the cholesterol: phospholipid ratio showed a significant difference between the grades at the level $P = 0.01$. Using only the muscular arteries as the criterion, a similar but less significant difference appeared between the grades in the cholesterol level.

Comment. These observations show that at least total serum cholesterol and the cholesterol: phospholipid ratio reflect the activity of atherogenesis. Previously, *Stamler* and *Katz* (1950), using a very slightly atherogenic diet, observed a similar association between the plasma cholesterol level and atherogenesis in the thoracic aorta, but not between the latter and the ratio cholesterol: phospholipid. They found no relationship between the above-mentioned lipid values and atherogenesis in the muscular aorta.

Effect of Coccidiosis on the Results of the Experiment

Coccidiosis appeared in the experimental animals a week before the experiment, at the beginning of the second week of the experiment and at the end of the fourth week, and the birds were consequently given sulphonamide therapy during the week before the experiment, and during the second and third weeks, and chlortetracyclin during the fifth and sixth weeks of the experiment (Fig. 13).

In all the experimental groups the probability of contracting the infection was equally great, and there was no difference

Fig. 13. Changes in the serum lipid values during the experiment, and the periods of sulphonamide and chlortetracyclin therapy. Active chol.-fed group - - o - -, inactive chol.-fed group ——•——, active control group - -o- -, and inactive control control group ——•——.

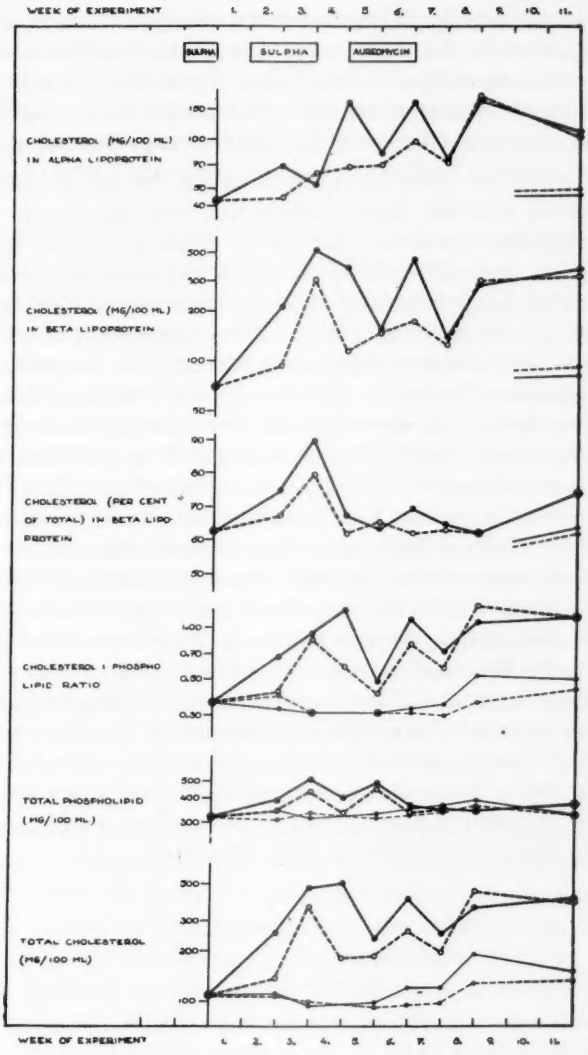
WEEK OF EXPERIMENT
1. 2. 3. 4. 5. 6. 7. 8. 9. 10. 11.
SULPHA
SULPHA
AUREOMYCIN
CHOLESTEROL (MG/100 ML) IN ALPHA LIPOPROTEIN
CHOLESTEROL (MG/100 ML) IN BETA LIPOPROTEIN
CHOLESTEROL (PER CENT OF TOTAL) IN BETA LIPOPROTEIN
CHOLESTEROL : PHOSPHOLIPIC RATIO
TOTAL PHOSPHOLIPID (MG/100 ML)
TOTAL CHOLESTEROL (MG/100 ML)
WEEK OF EXPERIMENT
1. 2. 3. 4. 5. 6. 7. 8. 9. 10. 11.

Fig. 13.

between the groups in morbidity and mortality; thus it seems unlikely that the incidence or severity of the disease differed in the various groups. Where treatment was given, it was always administered in the same manner to all the experimental animals. Consequently, it seems quite impossible that the coccidiosis or its treatment had any effect on the differences found in the experiment between the different experimental groups.

It is, on the other hand, impossible to be certain how far coccidiosis and the sulphonamide and chlortetracyclin therapy employed influenced the serum lipid levels and atherogenesis in general. Sick cockerels eat less than healthy birds, and in intestinal infections in general resorption is decreased. Thus it seems likely that the diseased experiment animals in fact get less cholesterol than the healthy animals. Sulphonamide may increase the serum lipid levels, the cholesterol level at least, *via* inativation of the thyroids (*Franklin* and *Chaikoff 1943, Schachner* et al. 1944). *Nelson* et al (1953) noticed that chlortetracyclin enhanced atherogenesis and raised the plasma cholesterol level in rabbits. Comparison of the times of therapy with the fluctuations of lipid values reveals that sulphonamide therapy was in use during the rapid rise of lipid levels of the cholesterol-fed groups in the second and third weeks of the experiment. But against the assumption that this contributed to the rise is the fact that no rise was found in the control groups during this period. Chlortetracyclin therapy coincided with the post-peak period of the cholesterol-fed groups, which shows that it had, at least in present experimental conditions, no influence on the serum lipids. It does seem unlikely that this complication of the experiment had any influence at all on the serum lipid levels and, consequently, on atherogenesis.

Discussion

The experiment showed that physical activity had a distinct effect on cholesterol-induced atherosclerosis in cockerels. Both the incidence and severity of the disease proved to be considerably greater in inactive cockerels confined in small cages than in the active birds permitted to move about freely in the pen but kept on the same diet and in similar conditions otherwise.

The effect of physical activity on experimental atherosclerosis has been studied previously but little. *Brown* et al. (1956) studied the effect of exercise on the development and disappearance of atheromatosis in rabbits, but failed to show any differences between the exercise and control groups. The method they employed, however, was unlike that used in the present experiment. In their study, the difference in physical activity between the groups was produced by artificially increasing the activity of the exercise group, whereas here the difference was caused by artificially restricting physical activity. It seems improbable that the different means of varying activity are comparable. If the aim is to throw light *via* animal experiments on the factors affecting atherogenesis in man — which is naturally an important consideration in experimental pathophysiology — the method used in the present study answers the purpose better than methods based on an artificial increase in physical activity. Indeed, the decades during which coronary artery diseases have become very common have also seen a distinct change in human living towards less physical exertion as mechanisation has grown, the »white collar» class increased and means of communication improved. The method used by *Brown* et al. also differs in another respect from the present one. *Brown* et al. increased the physical activity of the active groups daily by exercise of short duration, 20 minutes at a time. In the present work, on the other hand, the difference in activity was continuous, just as it is with humans in different occupations. In this respect, too, the methods are probably not comparable.

On the other hand, *Wong* et al. (1956) reported results which showed that atherosclerosis of the abdominal aorta was less severe in cholesterol-fed chickens exercised twice daily than in birds which were not evercised. The diet contained 2 per cent of cholesterol and the experiment lasted eight weeks. The methods used in the investigation were not published. Therefore it is difficult to conclude whether the methods used by *Brown*'s and *Wong*'s groups are comparable. If they are comparable, the divergent results possibly indicate a different behaviour between the species, rabbis and chickens. The results obtained by *Wong* et al. are in agreement with the results of the present study; both investigations showed that the cholesterol-induced atherosclerosis was less severe in more active chickens or cockerels than in less active birds.

Activity was found to have no effect on the development of atheroslerosis in cockerels on the control diet. *Wolffe* and his associates have reported investigations which suggested that physical activity probably affects spontaneous atherosclerosis in geese. They (*Wolffe* et al. 1949) found that the incidence of atheromatosis was much smaller in wild ducks than in fattened geese reared in captivity, and they suggested the difference in physical activity as a possible explanation for the observation. The birds studied were, however, not of the same breeds; there were also other differences and, consequently, no reliable conclusion is warranted from the observation mentioned as to the effect of physical activity. In another, more precisely documented investigation, *Wolffe* and his associates (1952) found that of eight geese kept in small cages and fed with common poultry feed, two developed mild atheromatous changes of the aorta in three months. The investigators regarded confinement as one factor producing atherosclerosis in these cases. The geese were 11 months old at the end of the experiment, by which age they may have spontaneous atherosclerosis. Hence, owing to the smallness of the material the result may be due to mere chance. It is thus impossible to obtain a reliable idea of the effect of physical activity on spontaneous atherogenesis from

investigations made so far, and the experimental animals used in the present study were obviously too young to elucidate this point.

In the inactive cholesterol-fed group the levels of total cholesterol, phospholipid, the ratio cholesterol: phospholipid, the cholesterol contents of the alpha and beta lipoproteins and the percentage of total cholesterol in the beta lipoprotein fraction were significantly higher than in the active cholesterol-fed group during the greatest part of the experiment; at least up to the beginning of the eighth week of the experiment which lasted 10—12 weeks in all.

The observations indicate that physical activity inhibits, or physical inactivity enhances, the rise in the serum lipid values of cockerels which are under the influence of an atherogenic factor, in this case cholesterol feeding. This agrees with many observations made earlier on both man and experimental animals. Comparisons of populations differing in their physical activity have revealed a lower plasma cholesterol level in active populations or human groups than in inactive populations. Some attribute the difference chiefly to the difference in physical activity (*Mann* et al. 1955 a and b, *Chailley—Bert* et al. 1955), while others are of the opinion that physical activity has but a slight effect (*Keys* et al. 1956 a). In man, too, physical activity has been found to prevent an increase otherwise occurring in the level of cholesterol and other lipids (*Mann* et al. 1955 c, *Keys* et al. 1956 b). Sudden, strenous physical exercise, on the other hand, seems either to raise the plasma cholesterol level (*Fahrig* and *Wacker* 1932) or to have no effect on it (*Rakestraw* 1921, *Patterson* 1927), and to increase as a rule the phospolipid level (*Gage* and *Fish* 1924, *Patterson* 1927, *Stewart* et al. 1931, *Fahrig* and *Wacker* 1932). However, the effect of sudden, strenuous exercise cannot be compared with the difference in physical activity produced in the present study by limiting the activity. No animal experiments comparable in the methods employed have been made. The results of the investigation made by *Brown* et al. (1956) showed that of the rabbits kept on a cholesterol

diet those that were subjected daily to compulsory exercise of 20—60 minutes had towards the end of the experiment serum cholesterol values which were only about a half of the values of the non-exercise group. *Wong* et al. (1956), who observed that exercise inhibited atherogenesis in cholesterol-fed chicken, did not find any difference in blood cholesterol levels between the exercise and non-exercise groups. Blood samples probably were taken at the end of the eighth week of the experiment, at which time the difference in cholesterol values between the active and inactive groups had disappeared also in the present study. *Peltonen* and *Karvonen* (1956) did not find in their experiments on mice that compulsory swimming affected the serum cholesterol level.

Physical activity was not found to affect the plasma lipid levels of cockerels kept on a normal non-atherogenic diet.

The present investigation showed that at least the level of total cholesterol and the ratio cholesterol: phospholipid to some extent reflect the degree of atherogenesis. The lipid values associate clearly and significantly with the severity of the atheromatosis in the elastic vessels, more uncertainly with that in muscular arteries. These observations are confined to cockerels fed on cholesterol; had the cockerels on control diet been included the association between the lipid values and the development of atherosclerosis would naturally have been exceedingly clear. As information on this point is based on lipid values at the end of the experiment, at which time the lipid values for the active and inactive cholesterol-fed groups did not differ, the result cannot be due to the effect of physical activity but is in fact evidence of a positive association between atheromatosis and the lipid values. Previously *Stamler* and *Katz* (1950) found that the severity of atherosclerosis in chicken on a very slightly atherogenic diet correlated to some extent with the serum cholesterol values obtained during the experiment. Furthermore, a study where the effects of different amounts of dietary cholesterol feeding were observed indicated that the duration and degree of hypercholesterolemia were in positive correlation with the severity of atherosclerosis (*Horlick* and *Katz* 1949).

Judging from previous investigations in part and from the present study, at least the total cholesterol level and the ratio cholesterol: phospholipid are associated positively with the development of atherosclerosis at least in chicken and obviously in man too. This being so, two observations made in the present work show close conformity: the incidence and severity of atherosclerosis were greater in the cholesterol-fed inactive group, and the serum lipid values studied were higher during the experiment in that group than in the active cholesterol-fed group of cockerels.

The study in which the functional activity of the thyroids was determined by the quantitative histological method showed that in the cholesterol-fed cockerels the thyroid activity was lower in the inactive than in the active group. This is especially interesting as the thyroid hormone is known to influence both the development of atherosclerosis and serum lipids. The effect is very distinct in the chicken. The Michael Reese group found that the thyroid hormone has a marked inhibitory effect on both the rise in serum cholesterol and the development of atherosclerosis in cholesterol-fed (*Dauber* et al. 1949, *Stamler* et al. 1950 b) as well as in stilbestrol-treated (*Stamler* et al. 1950 a) chickens.

From information available about the effect of thyroid hormone on atherogenesis and serum lipids in chicken it is obvious that the highly significant difference in thyroid activity observed between the active and inactive cholesterol-fed groups may explain the differences in atherosclerosis and serum lipid values between these groups. This conclusion is quite correct in spite of the fact that from the present study it is impossible to decide what really are the factors which influence thyroid activity. It is impossible, however, to conclude whether the difference in thyroid activity was so great that it could account entirely for the marked differences in serum lipid values and atherogenesis between the active and inactive cholesterol-fed groups.

The present study clearly shows that physical inactivity enhances atherogenesis and the rise of serum lipid values in

cholesterol-fed cockerels. Physical inactivity had this effect at least in part *via* inactivation of the thyroids. Disturbances in caloric balance, as far as body weight is an indication, were not concerned, for the active and inactive groups did not differ in body weight.

Especially in respect to the mode of action of physical activity or inactivity this study leaves many questions open and calls for further investigations.

SUMMARY

The main purpose of the present investigation was to study the effect of physical activity on atherogenesis in the aorta and in the brachiocephalic and iliac arteries, and on serum lipid values in cockerels on commercial poultry diet and in cholesterol-fed cockerels. At the beginning of the experiment the cockerels were about seven weeks of age. The experiment lasted 10—12 weeks. The cholesterol diet contained 1.5 per cent of cholesterol. The difference in physical activity was brought about by artificially limiting the activity in the inactive groups, confining the cockerels individually in small cages. The physically active groups were allowed freely to move in large pens. In other respects the conditions were the same. The values of serum total cholesterol, phospholipid, the cholesterol: phospholipid ratio and the content of cholesterol in the alpha and beta lipoproteins, fractionated by paper electrophoresis, were determined before the experiment, at weekly intervals during the experiment from birds selected at random from each group, and at the end of the experiment. With the object of obtaining some information about the *modus operandi* of physical activity, body weights and the thyroid activity of cockerels on the different experimental groups were compared, the latter values being estimated by a quantitative histological method.

The main results were as follows:

1. No difference appeared either in the serum lipid values or atherogenesis between the active and inactive control groups fed on ordinary diet.

2. In the cholesterol-fed groups the lipid values of the inactive group were significantly higher than those of the active

cholesterol-fed group during the greatest part of the experiment, at least up to the beginning of the eighth experimental week. In this respect no difference was found at the end of the experiment.

3. The incidence of atherosclerosis was higher and its severity more marked in the inactive cholesterol-fed cockerels than in the active cholesterol-fed group.

4. The body weight of the active and inactive groups did not differ.

5. The thyroid activity of the inactive cholesterol-fed group of cockerels was significantly lower than that of the active cholesterol-fed cockerels.

The observations that the serum lipid values during the experiment were higher and, on the other hand, atherogenesis more marked in the inactive cholesterol-fed cockerels than in the active birds concur well. Since thyroid hormone has been shown to have a very distinct effect both on some serum lipid values and on atherogenesis in cholesterol-fed cockerels the above-mentioned differences between the active and inactive cholesterol-fed groups were interpreted as being, at least partly, a result of thyroid inactivation in the inactive cholesterol-fed group.

Besides the findings connected with the main problems of the study observations were made that may be of some interest. The cholesterol content of alpha lipoprotein, too, increased, especially during the latter half of the experiment. This does not agree with observations made on man. No increase in cholesterol content in human alpha lipoprotein has been observed in association with atherosclerosis or in hypercholesterolemic conditions.

The values of total cholesterol and the cholesterol: phospholipid ratio at the end of the experiment correlated positively with the severity of atherosclerosis in the elastic vessels; in the muscular arteries the correlation was almost significant.

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